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The role of iron and iron chelators in zygomycosis

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Abstract

Iron is an essential element for cell growth and development, contributing to DNA synthesis and regulating the G_1-phase to S-phase transition. Moreover, iron is important for the virulence of the majority of microorganisms, and the function of the genes regulating iron uptake is coupled with the manifestations of the virulence phenotype. All fungi elaborate specific uptake mechanisms to sequester iron, and most commonly produce small molecules with high affinity for iron, the siderophores. The importance of iron appears to be particularly high for Zygomycetes, which grow abundantly in iron-rich media, and all the known predisposing factors for zygomycosis have, as a common feature, the increased availability of free iron. Among the known iron chelators, deferoxamine supports the growth of Zygomycetes because it acts as xenosiderophore, delivering iron to iron-uptaking molecules of these species. Conversely, the newer iron chelators deferiprone and deferasirox do not exhibit similar activity, apparently because they share higher affinity constants for iron and, as a result, deprive the fungi of iron, inhibiting their growth. This activity has been documented in various culture systems and in many animal models of zygomycosis, and therefore suggests that these drugs might be used as adjuvant treatment for systemic zygomycosis. There are few case reports in which the newer iron chelators have been used as antifungals, and their possible benefit must be verified in a prospective randomized trial.

Keywords: Fungal growth, iron, iron chelation, treatment, zygomycosis

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Introduction

Iron is a key element for the growth and development of any cell or organism. Eukaryotic cells, entering the S-phase of the cell cycle, upregulate transferrin receptor-1 expression to obtain iron from the extracellular environment. Iron is a cofactor of ribonucleotide reductase, which is necessary for DNA synthesis, and of the oxygen transporters in mitochondria, and activates the cyclin/cyclin-dependent kinase complexes, thus regulating the progression from the G_1-phase to the S-phase of the cell cycle.

The Role of Iron in Cell Growth

Low levels of intracellular Fe^{3+} increase the activity of the cyclin-dependent kinase inhibitor p21CIP1/WAF1, thus delaying or inhibiting transition to S-phase. As a result, Bcl-2 is downregulated and Bax levels are increased, conditions that activate caspase-3, caspase-8, and caspase-9, and lead to apoptosis and cell death [1].

Iron is essential for the virulence of all infectious microorganisms. The low concentration of free iron (non-transferrin-bound iron) in plasma and other tissues represents a major protective mechanism against invading pathogens. An extremely low level (10^{-18} M) is maintained because of the action of two major iron-binding proteins, transferrin and lactoferrin, which are partly saturated with iron (usually ~30% and, in general, <50%) and seek to incorporate ferric iron [2]. Lactoferrin is produced and released by many cell types, including neutrophils and monocytes, and represents the fungistatic factor of human serum, milk, and other fluids [3]. Pathogens require 10^{-6} to 10^{-7} M iron for growth, and, therefore, serum and other biological fluids are normally fungistatic for all fungi, including *Candida* spp., *Aspergillus* spp., and the Zygomycetes [4]. The fungistatic properties of human serum *in vitro* are completely abolished by the addition of exogenous iron, and *Candida albicans* is capable of growth in serum cultures with transferrin saturation >90%, but not when serum has normal transferrin saturation.

Thus, pathogenic microorganisms have developed very sophisticated methods to obtain, or rather 'steal', iron from their hosts, establishing a strong competition for iron with

them. The outcome of the 'iron battle' will determine whether there will be a clinically apparent infection, a carrier state, or elimination of the invader. Diseases and conditions accompanied by a high iron burden have been associated with increased susceptibility to infections. Among these are tissue hypoxia, diabetic ketoacidosis, acidosis of any other cause, tissue damage and necrosis, post-traumatic states or those induced by chemotherapy, haemochromatosis, liver disease, and cancer. There are three sources of iron for microbial growth: proteinic iron from the iron-carrying proteins (ferritin, transferrin, etc.), haem iron following lysis of red blood cells, and tissue iron, which is available following tissue damage [2,4].

Patients with acute myelogenous leukaemia or other malignancies commonly have an excess of iron, as demonstrated by elevated serum transferrin saturation. The levels of iron and, particularly, non-transferrin-bound iron are further increased following chemotherapy [5], either because of tissue damage or, in some cases, as a result of circulating iron complexes. The complexes are produced by the leukaemic cells and liberated following their death, induced by chemotherapy. All of the above render leukaemic neutropenic patients particularly vulnerable to fungal infections. *C. albicans*, for example, grows readily in leukaemic serum with high transferrin saturation. Chemotherapy also elevates free serum iron levels in patients with gastric cancer and non-Hodgkin's lymphoma [4].

Finally, liver iron overload in patients undergoing orthotopic liver transplantation is a predisposing factor for the development of invasive fungal infections, as these infections occur three times more commonly among transplanted patients with sustainable levels of iron in the liver [6].

Iron and Fungal Development

Iron uptake by fungi is achieved by specific transport systems, in which an initially ferric form is reduced to ferrous iron through the action of specific cell surface reductases (ferroxidases). Ferrous iron is then internalized by three different mechanisms. The first depends on the fact that iron-containing ferroxidases have a high affinity for a specific type of fungal transport proteins, the permeases. The iron permease–ferroxidase complexes (Ftr1–Fet3) easily transverse the fungal wall and cell membrane, and iron is therefore provided intracellularly. There are specific transcriptional activators or repressors of the genes of the ferroxidases and permeases, such as Aft1 and Aft2 in *Saccharomyces cerevisiae* or the *Cryptococcus* iron regulator gene (*CIR1*) in *Cryptococcus neoformans*, which modulate their expression under conditions of iron deprivation. A second mechanism involves the production of low molecular mass iron-specific chelators (siderophores), which are excreted through the fungal wall in the deferric form, bind iron, and then are taken up by the fungi. Finally, a third mechanism is related to a fungal haem oxygenase, which takes up iron from haem [7,8].

C. albicans possesses two high-affinity iron permease genes that are essential for its virulence. Iron permeases are encoded by iron-responsive genes, which are regulated by specific transcriptional modulators. Deletion of these genes renders mutant strains non-virulent [9]. Conditions of iron overload enhance the growth of *C. albicans* and increase the mortality rate of infected mice. Elevated serum iron levels have been documented among patients with urogenital candidiasis. *CIR1* is a gene regulating iron homeostasis, but also regulates calcium and cAMP signaling, cell wall integrity, and the expression of all virulence functions, including capsule and melanin formation and growth at host temperature. Non-enzymatic reduction of ferric iron by 3-hydroxylanthranilic acid and melanin has been documented in *Cryptococcus neoformans* [10].

The expression of permease genes in *Aspergillus* spp. and zygomycetes is upregulated during their growth and virulence [11]. The growth and survival of *Aspergillus fumigatus* and other species in serum is associated with the removal of iron from transferrin and other iron-containing proteins [12]. This is accomplished by the activity of siderophores.

There are two major classes of microbial siderophores, a list of which is provided in Table 1. Fungal species are capable of synthesizing many different siderophores; however, the most important and most commonly found in *Aspergillus* and zygomycetes are *N′*,*N′*,*N*-triacetylfusarinine C and ferricrocin. These substances exhibit extremely high affinity for iron, and hold it with three bidentate bonds. The high affinity is specific for iron, and does not extend to other bivalent cations. The production of siderophores is enhanced in conditions of iron depletion, and many metabolic steps of their

TABLE 1. Bacterial and fungal siderophores

Hydroxamate class siderophores
N′,*N′*,*N*-triacetylfusarinine C*
Rhodorotulic acid
Aerobactin‡
Enterobacin‡
Ferrichrome class siderophores
Ferricrocin#
Ferrirubin
Ferrichrysin
Ferrirhodin
Asperchromes
Ferrichrome
Fusigen

*Most common, #second most common, ‡bacterial siderophores.

biosynthesis have been characterized. Siderophores have higher binding constants for iron than transferrin and lactoferrin, and thus are capable of detaching iron from these proteins. Their biosynthesis is confined to bacterial and fungal cells, and their expression increases the virulence of these species [13]. *Aspergillus* uses two iron uptake mechanisms, the reductase–permease complex and the siderophore-assisted mechanism [14]. The latter has been demonstrated *in vitro*, as holotransferrin, but not apotransferrin, supports the growth of *Aspergillus* spp. in iron-depleted serum culture systems. In such systems, siderophore production becomes evident following 10 h of incubation and reaches a peak at 20 h [12].

Nevertheless, not all species and strains produce siderophores. Some fungi use ferric reductases or low molecular mass iron reductants to reduce ferric to ferrous iron, and extract it from the extracellular environment. Such mechanisms have been documented in *C. albicans*, *Histoplasma capsulatum* [15], and *Cryptococcus neoformans* [16].

Zygomycetes and Iron

Zygomycosis is a difficult-to-treat systemic fungal infection, caused by Zygomycetes, with a high mortality rate, ranging from 50% to 100%. The disease in any clinical form is characterized by the propensity of Zygomycetes for vascular invasion and dissemination, commonly resulting in thrombosis and tissue necrosis. The infection can rapidly extend from the paranasal sinuses to the oral cavity, to the orbit, and intracranially, sometimes producing cavernous sinus thrombosis [17]. Zygomycosis almost always occurs among patients with a pre-existing immune defect, although rare cases have been reported among apparently normal individuals [18]. In the majority of cases, the disease is rapidly progressive and eventually fatal, unless prompt treatment with high doses of liposomal amphotericin B (LAmB), in association with careful surgical debridement, can change the otherwise dismal clinical course. Because the spectrum of diseases, for which immunosuppressive treatments are used, has enlarged, the application particularly non-myeloblative preparatory regimens is increasing [19] and systemic antifungal prophylaxis with agents, ineffective against zygomycetes is broadened, the incidence of zygomycosis appears to have increased in the last few decades [20].

Well-recognized predisposing factors for zygomycosis are diabetes mellitus (especially when complicated by ketoacidosis), treatment with corticosteroids, immunosuppression, prolonged leukopenia (neutropenia and lymphopenia), recent chemotherapy and tissue damage, history of allogeneic stem cell transplantation, and chronic graft-versus-host-disease. A common denominator of almost all of these conditions is the presence of excessive iron overload, as a high tissue iron burden, as elevated serum transferrin, and also as increased non-transferrin-bound iron. In particular, it has been suggested that diabetic ketoacidosis and acidoses of any aetiology predispose to zygomycosis by facilitating the dissociation of iron from iron-carrying proteins, thus providing increased available free iron [21]. Elevated serum and tissue iron have a tremendous impact on the growth and development of Zygomycetes [22]. There are reports of fast *Mucor* growth with formation of intra-arterial thrombi among immunocompromised patients with iron overload [23,24]. In a retrospective analysis of 263 allotransplanted patients, five cases of invasive zygomycosis were identified. In comparison with matched controls, these patients had significantly higher serum ferritin levels, transferrin saturation, and number of previously transfused red blood cell units. It is of note that Zygomycetes possess a specific high-affinity iron permease gene (RFTR1), which has been characterized and cloned [25]. Analysis of the polymorphisms of this gene, performed by using a combined PCR amplification and sequencing method, has recently been proposed as a tool for the molecular identification of the different Zygomycete species [26].

The Role of Deferoxamine (DFO)

The well-documented and repeatedly reported increased susceptibility to zygomycosis of haemodialysis patients during treatment with DFO, an iron chelator that is capable of removing tissue iron, initially appeared to be a paradox [27,28]. It became clear, however, that although DFO chelates iron, from the perspective of Zygomycetes it is a xenosiderophore, as fungal siderophores have higher affinity for iron than DFO and therefore are capable of easily and effectively detaching iron from it and providing it to the fungi [7,29]. This ability is particularly prominent in Zygomycetes, and these species can remove eight and 40 times greater amounts of iron from DFO than *A. fumigatus* and *C. albicans*, respectively.

The rapid and effective iron uptake by Zygomycetes results in rapid growth in serum. The growth of *Rhizopus rhizopodiformis* spores, isolated from a dialysis patient with zygomycosis while on DFO therapy, was studied in an iron-deficient medium containing human serum at increasing concentrations and with human serum enriched with different concentrations of ferrioxamine (DFO–iron complex). A concentration of 40% human serum inhibited fungal growth by >50%. However, in the presence of serum, ferrioxamine produced significant

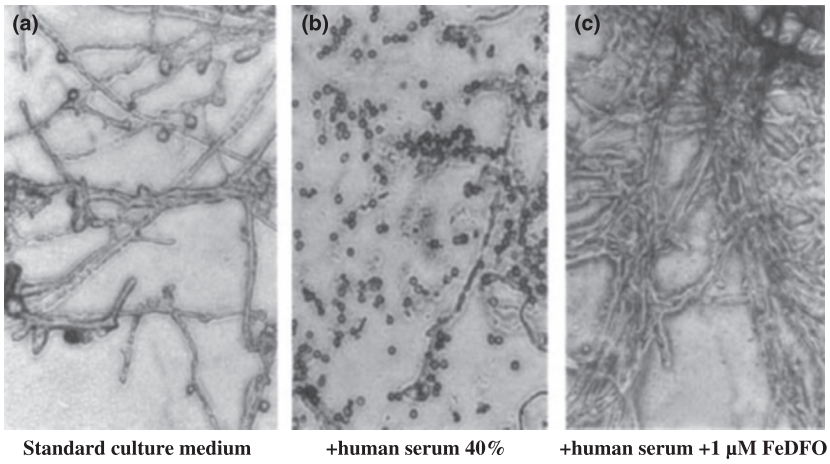

FIG. 1. Effect of human serum and of ferrioxamine on the growth of *Rhizopus microsporus*. Spores of *Rhizopus* were cultivated for 24 h at 37°C in standard BDM culture medium alone (a), in BDM with 40% human serum (b), or in BDM with 40% serum + 1 μM Fe deferoxamine (Fe.DFO) (c). Lugol stain ×500. Reprinted from reference [29].

growth stimulation at 24 h that persisted at 48 h [29]. The effect of human serum and of ferrioxamine on the growth of *Rhizopus* found in this study is demonstrated in Fig. 1. Data from animal models emphasize the exceptional requirement of iron for *Rhizopus* pathogenicity, as administration of DFO or free iron worsens the survival of animals infected with *Rhizopus*, but not with *Candida* [30].

DFO has been demonstrated to act as a xenosiderophore in *Rhizopus*, other members of the Mucorales, and probably other pathogenic fungi. It is assumed that fungal enzymes or siderophores are able to specifically bind to ferrioxamine and, because they have higher affinity for iron, strip iron from ferrioxamine and facilitate iron uptake by the fungi. A similar phenomenon does not take place with deferiprone [31]. The susceptibility to zygomycosis of dialysis patients treated with DFO could be attributed to the facts that uraemia results in significant retention of the iron-loaded ferrioxamine in the circulation, and that this is removed during dialysis, causing patients' serum to lose its fungistatic power and be transformed to a favourable culture medium for Zygomycetes [32].

The Role of Newer Iron Chelators

There are two newer iron chelators in general use, deferiprone (DFP) (Ferriprox, DFP; Apotex, Toronto, Canada), which was introduced in the 1990s, and deferasirox (DFX) (Exjade; Novartis–DFX, Basel, Switzerland), which was introduced more recently. Both drugs are effective iron chelators in clinical practice, but their use has not been associated with increased numbers of fungal infections or, particularly, of zygomycoses. The reason for this discrepancy, as compared with DFO, may be the different chemical structures and chelating affinities of the three drugs. DFO is an exadentate chelator, has a higher molecular mass, and shows a molecular chelating relationship with the ferric iron of 1 : 1, which implies that each DFO molecule chelates one ferric ion. DFP is a bidentate chelator, and its chelating relationship is 3 : 1, meaning that each ferric iron is chelated by three molecules of DFP. DFX is a tridentate chelator, and its chelating relationship is 2 : 1, meaning that each ferric iron is chelated by two molecules of DFX) [33]. The chemical structures of the three iron chelators are shown in Fig. 2.

The two newer iron chelators do not act as xenosiderophores, apparently because the fungal iron uptake systems are incapable of detaching iron from them. This could be due either to inadequate molecular access, as they are smaller molecules than DFO, or to their higher affinity for iron, which means that DFP and DFX might form more stable chemical structures with iron that are not destabilized in the presence of fungal enzymes or siderophores. Moreover, the demonstration of clear inhibitory activity of the two newer chelators on fungal growth suggests that these molecules are probably capable of detaching iron from the fungal iron uptake molecules and holding it more strongly. This has been proven *in vivo*, using animal models of zygomycosis, in which treatment of *Rhizopus*-infected mice or guinea pigs with DFP markedly improved survival [31]. In cultures of *Rhizopus oryzae*, DFP has fungistatic activity at 24 h, confirmed at 48 h [34].

The introduction of DFX and the recognition of the safety and efficacy profile of the drug encouraged its use in sporadic cases of systemic zygomycosis and in experimental animal studies. DFX induces an iron-starvation response in *R. oryzae* and activates *RFTR1* expression. Addition of DFX to cultures of different members of the Mucorales produced a fungicidal effect, which was reversed by the addition of iron. The MIC_{90}s of DFX against various *Mucor* spp. were much lower than the levels achieved by the administration of the usual daily dose of 20 mg/kg. Treatment with routine

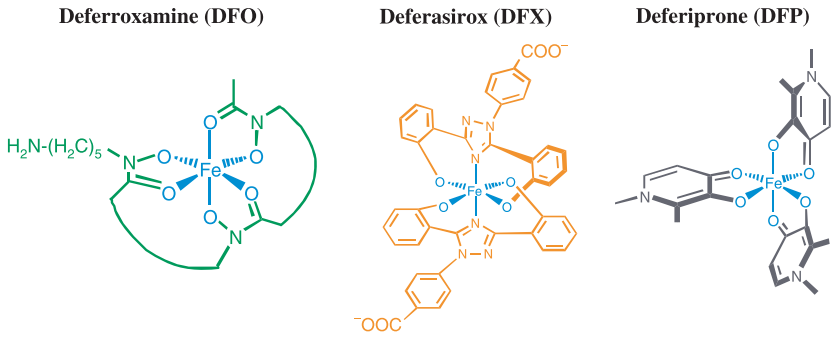

FIG. 2. Stereochemical structure of the three available iron chelators. Stereochemical structures and molecular chelating relationships of the three available iron chelators. Deferoxamine (DFO) has a higher molecular mass and is a hexadentate chelator; that is, each molecule holds one ferric iron (chelating ratio 1 : 1). Deferasirox (DFX) has a lower molecular mass and is a tridentate chelator; that is, two DFX molecules hold each ferric iron (chelating ratio 2 : 1). Deferiprone (DFP) has an even lower molecular mass and is a bidentate chelator; that is, three DFP molecules chelate each ferric iron (chelating ratio 3 : 1).

doses of DFX of diabetic ketoacidotic mice infected with spores of *R. oryzae* led to significantly improved survival as compared with controls, and resulted in a more than ten-fold reduction of brain and kidney fungal burden as compared with placebo-treated animals. The kidneys of DFX-treated mice had no visible hyphae and there was an effective neutrophil inflammatory reaction, whereas kidneys of placebo-treated mice had extensive filamentous fungi and manifested a poor or complete absence of a neutrophil inflammatory response [35].

In another experiment, mice infected intranasally with 10^7 spores of *R. oryzae* were treated for 7 days, starting 24 h post-infection, with either DFX 10 mg/kg twice daily or placebo. As controls, infected or uninfected mice were treated with DFO 50 mg/kg. DFX was significantly more protective than placebo or DFO. As expected, DFO worsened the survival of infected mice, although it had no effect on uninfected mice (Fig. 3). Treatment with DFX resulted in significantly increased Th1 and Th2 splenocyte subpopulations, and in significantly higher splenic and kidney levels of the proinflammatory cytokines tumour necrosis factor-α and interferon-γ, than those in mice treated with saturating iron or placebo [35].

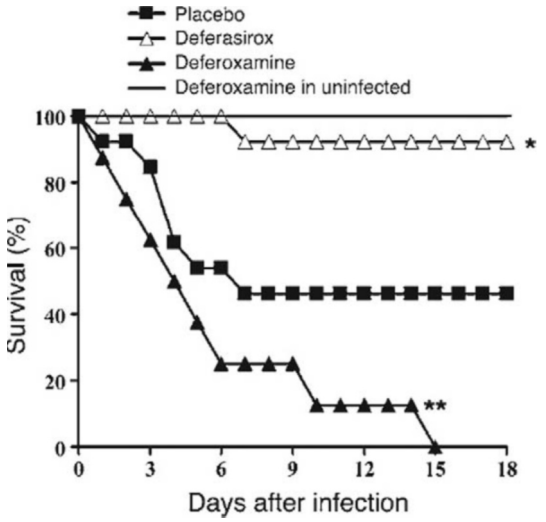

FIG. 3. Effect of deferoxamine (DFO)and deferasirox (DFX) treatment on the survival of mice infected with *Rhizopus*. Survival of diabetic ketoacidotic mice infected intranasally with 10^7 spores of *Rhizopus oryzae* and 24 h later treated with placebo (n = 13), DFX 10 mg/kg twice daily (n = 13), or DFO 50 mg/kg (n = 8). DFX protected the animals as compared with placebo-treated mice ($p < 0.047$) or DFO-treated mice ($p < 0.009$). Reprinted from reference [35].

Iron Chelators as Adjuvant Treatment in Systemic Zygomycosis

In a mouse model of zygomycosis, animals were infected with *R. oryzae* spores, and 24 h later were treated with DFP at dose levels of 50, 100 or 200 mg/kg every day or every other day. The dose of 100 mg/kg every other day resulted in a significant survival advantage in DFP-treated mice as compared with placebo-treated animals. The other dose schedules were either ineffective or toxic. The survival advantage was comparable to, although lower than, that of LAmB-treated mice. Both drugs significantly reduced the brain fungal burden as compared with placebo. The beneficial effect of DFP was abrogated when animals were given ferric chloride [34]

In a similar mouse model with established zygomycosis, the administration of DFX was associated with efficacy comparable to that of LAmB. DFX has shown efficacy in neutropenic and diabetic ketoacidotic mice with zygomycosis. In these experiments, DFX at a daily dose of 20 mg/kg initiated 24 h after infection was synergistic with LAmB at a high-dose schedule of 15 mg/kg daily in the reduction of

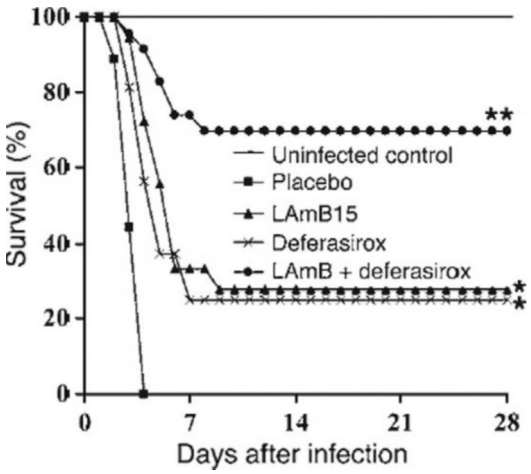

FIG. 4. Demonstration of synergistic activity of deferasirox (DFX) and liposomal amphotericin B (LAmB) against *Rhizopus oryzae*. Survival of diabetic ketoacidotic mice infected with *R. oryzae* and treated with DFX 10 mg/kg twice daily for 7 days, or LAmB 15 mg/kg for 4 days, or a combination of both, or placebo. DFX exhibited protective activity similar to that of LAmB, and the combination of both rescued a significantly higher number of mice. Reprinted from reference [35].

fungal burden from the brain and the kidney. Moreover, the combination of the two drugs significantly improved survival time as compared with placebo or each drug separately (Fig. 4) [35].

The use of iron chelators as adjuvant treatment in systemic zygomycosis and other mycoses appears to be rational, and has been shown to be effective in sporadic cases. Reed *et al*. reported a case of a 40-year-old diabetic patient with aggressive rhinocerebral zygomycosis and progressive central nervous system involvement, despite combination treatment with high-dose LAmB plus caspofungin and surgical debridement [36]. As brain magnetic resonance imaging showed new parenchymal lesions and left cavernous sinus thrombosis, he was given a 7-day salvage treatment with DFX 1000 mg daily. A new brain magnetic resonance imaging scan showed significant improvement, and treatment with LAmB was discontinued. The patient, 4 months following treatment discontinuation, remained in good condition without any neurological deficit. This is the first reported case of zygomycosis being successfully treated with a combination of classical antifungal treatment and an iron chelator [36].

We recently treated a 37-year-old male with acute lymphoblastic leukaemia in remission following a matched-related allogeneic transplant, who manifested concurrent rhinocerebral and pulmonary zygomycosis, while undergoing treatment with corticosteroids for extensive chronic graft-versus-host disease. The patient was treated with a combination of LAmB 10 mg/kg, posaconazole and deferasirox 10 mg/kg twice daily. Restricted intranasal and intrasinus surgical debridement was also applied repeatedly. The patient responded very well, with rapid defervescence, resolution of pain and chymosis, and disappearance of the dense pulmonary and sinonasal infiltrates. He is now receiving weekly consolidation boosting of LAmB as outpatient therapy (A. Symeonidis, unpublished data).

In other cases, however, iron chelation treatment has been unsuccessful [37]. Therefore, the possible benefit of iron chelation as an adjuvant treatment in systemic fungal infections, and particularly in zygomycosis, has to be tested in a prospective randomized trial.

Conclusions

There are still many open issues for clarification: should iron chelation form part of the antifungal treatment in all systemic mycoses or only in zygomycosis? Will this treatment permit shortening of the duration of treatment with classical antifungal agents? Which are the best iron chelators and what are their optimal doses? Should systemic antifungal prophylaxis be administered to patients undergoing intravenous iron chelation therapy with DFO? Are some specific patient populations less or more prone to DFO-induced zygomycosis?

Currently, there is an ongoing prospective randomized trial, with the code number NCT00419770, introduced by the Los Angeles Biomedical Research Institute, the Deferasirox-AmBisome Therapy for Mucormycosis (DEFEAT Mucor Study). This study has been open since December 2007, and recruits patients with zygomycosis in an effort to answer some of the above-mentioned questions.

Transparency Declaration

The author is on advisory boards and has worked as a consultant for Gilead, Novartis and Celgene, has been on the speaker's bureau for Novartis and has received grant support from Genzyme and Actellion.

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